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Nutrition Bulletin: News from UK Research Councils

Early life dietary and epigenetic influences on childhood musculoskeletal health: the BBSRC component of the ERA-HDHL ALPHABET Project

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Abstract

The aim of the ongoing ALPHABET project, funded through the European ERA-HDHL Biomarkers call, is to expand our knowledge base regarding interactions between diet, epigenetics and offspring health, characterising biomarkers that may inform future health strategies. Each country partner is funded individually by a national funder. In this review article, we will briefly present an overview of the overall ALPHABET project, focusing in detail on the UK BBSRC funded component in which the aim is to 1) generate and collate early life epigenetic data and 2) investigate early diet and epigenetic marks as predictors of later bone health. The project builds on a wealth of evidence implicating environmental factors, such as maternal diet and body composition, as influences on the long-term health and development of the offspring, and that these relationships might be mediated at least in part through epigenetic signals. Thus, experimental studies in animal models have demonstrated that manipulation of maternal pregnancy diet leads to altered offspring epigenetic marking and phenotype. Human studies convincingly demonstrate associations between early environment and later health and disease for outcomes across musculoskeletal, respiratory, neurodevelopmental and cardiometabolic health. The priority now is to find ways in which such observations can be translated into improved lifelong health. A key approach is to identify early biomarkers of adverse health outcomes, and in the longer term, to test these, and subsequent interventions, in trials aimed at identifying strategies to optimise health throughout the lifecourse. The BBSRC funded component of the ALPHABET project permits us to inform this process for musculoskeletal outcomes, and the project as a whole should help elucidate not just novel mechanisms, but also potential strategies to reduce the burden of musculoskeletal, respiratory, neurodevelopmental and cardiometabolic disease in future generations.

Keywords (max. 6 words)

Maternal, nutrition, epigenetics, developmental, programming, bone

INTRODUCTION

The prevalence of obesity, cardiometabolic disease, asthma, osteoporosis and neurodevelopmental disorders have risen over recent decades. However, such increases in these non-communicable diseases cannot be fully explained by genetic or adult lifestyle factors – indeed there is increasing evidence to suggest that early life exposure to environmental factors, even before conception, influences health in older age (Godfrey et al., 2015b). The Developmental Origins of Health and Disease (DOHaD) hypothesis suggests that transient environmental exposures during critical periods of development (such as poor nutrition during fetal and early infant phases of life) can elicit lifelong effects on offspring health – through changes in gene expression both *in utero* and throughout life (Barker, 1995, Godfrey and Barker, 2000).

It is widely recognised that genes effectively provide a library of information that can be read (expressed) differently, in different cells and at different times, according to function and need. In a single organism, although the genetic code contained in every somatic cell is the same, the genes expressed will vary widely from organ to organ, and even from cell to cell, often in response to environmental cues (Gluckman et al., 2008). This regulation of gene expression involves a range of epigenetic processes whereby the DNA is modified to influence its expression, without changes in the genetic code itself. Epigenetic adaptations to suboptimal nutrition during pregnancy or early childhood may perpetuate changes in the health of the offspring many years after the exposure, and the consequences may even be seen in future generations (Burdge et al., 2011). Therefore, early life is a window in which interventions may help to prevent obesity, related cardiometabolic conditions and other chronic non-communicable diseases such as osteoporosis and asthma.

Modifications to maternal diet may be one such change in behaviour, which could play an important role in maternal, neonatal and child health outcomes. The urgent need to optimise health during early life, for example preconception/ during pregnancy and in the subsequent infancy of the offspring, has been emphasised by the World Health Organisation (WHO) in its comprehensive implementation plan on maternal, infant and child nutrition (2014b) and more locally by the WHO European Food and Nutrition Action Plan 2015-2020 (2014a). The WHO aims to achieve a 30% reduction in low birthweight babies and no increase in the prevalence of children who are overweight. Both low and high birthweight are associated with fetal and neonatal mortality and increased risk of later life non-communicable disease (Godfrey et al., 2015b).

Funded through the European ERA-HDHL Biomarkers call, the overarching aim of the ALPHABET project (led by Dr Catherine Phillips, University College Dublin) is to understand the specific dietary nutrients needed during pregnancy for optimal fetal growth and development and to identify

epigenetic processes responsible for linking maternal and childhood diet with future health and ageing. Whilst the full project will use data from a number of cohorts across Europe (see Appendix), and collaboration to generate novel dietary indices, this review will focus on the UK BBSRC-funded component of the overall project, the aim being to use the state-of-the-art in early life origins of bone health as an exemplar of the approach and the potential health benefits for the project as a whole.

EPIGENETIC MECHANISMS AND DEVELOPMENTAL PLASTICITY

Developmental plasticity, by which a single genotype may give rise to several different phenotypes in response to the prevailing environmental conditions, is found throughout the natural world. This process allows the next generation to be born appropriately adapted to the expected external environment, using cues from the organism's surroundings acting during critical periods of development (Hanson and Gluckman, 2014, Curtis et al., 2018b). A widely reported example is the meadow vole (*Microtus pennsylvanicus*), in which the thickness of the coat in the offspring is determined by the number of hours of light and dark experienced by the mother during gestation. Pups born in autumn have a thicker coat than those born in spring (Lee and Zucker, 1988), therefore adopting a developmental trajectory which is appropriate to the postnatal environment to which the meadow vole is likely to be exposed after birth. Maternal melatonin levels during pregnancy are the signal to the pup of the prevailing environmental conditions (Lee et al., 1989). However, it is easy to imagine how a mismatch between the expected postnatal environment and that to which the pup has been developmentally programmed would lead to a survival disadvantage (e.g. due to a change in the postnatal environment or inappropriate maternal cues) (Godfrey et al., 2007).

Various experimental studies have shown that alterations to maternal diet during pregnancy may lead to changes in offspring phenotype and gene expression (Lillycrop et al., 2005b, Burdge et al., 2007), and such effects may be underpinned by epigenetic mechanisms. They can be conserved across multiple generations but also can be reinstated *de novo* in each generation (Hanson and Skinner, 2016, Burdge et al., 2011). There is some evidence of associations between nutritional challenges in pregnancy and phenotypic effects on the grandchildren, although this does not prove a transgenerational effect as epigenetic effects can be induced in the primordial germ cells of the F1 during F0 pregnancy and produce effects in the F2 generation (Jaenisch and Bird, 2003, Grossniklaus et al., 2013). However, the epigenome can be regarded as a molecular record of life events, accumulating throughout a lifetime. Monozygotic twins are epigenetically most similar at birth, diverging with age, with the degree of divergence determined by the commonality of their environments (Fraga et al., 2005). It is clear that an understanding of these epigenetic processes has

the potential to enable early intervention strategies to improve child development and later health; as a consequence the study of epigenetic biomarkers is a rapidly advancing field (Godfrey et al., 2015a).

The three main epigenetic mechanisms are DNA methylation, histone modifications and non-coding RNAs, as summarised in Figure 1 (2008) (Gicquel et al., 2008) (Tang and Ho, 2007, Gluckman et al., 2008).

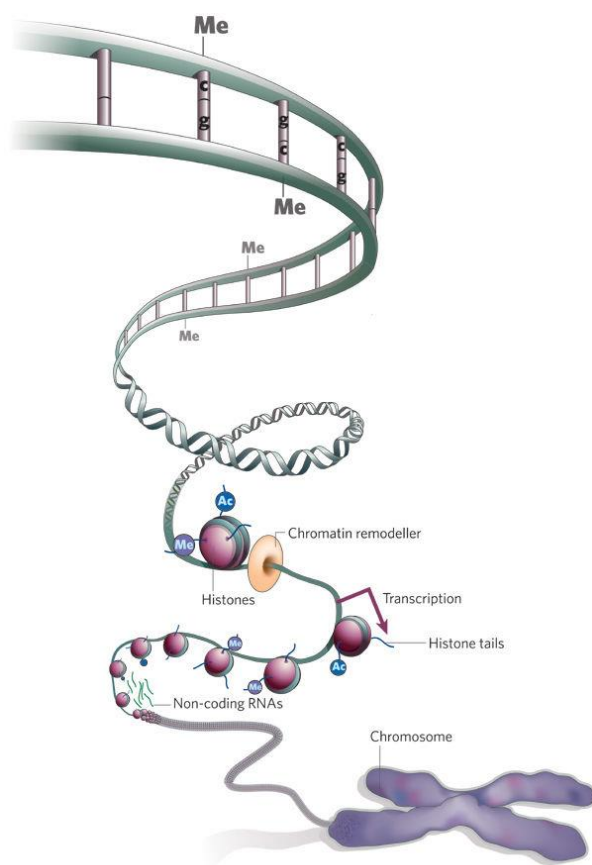


Figure 1: The main types of epigenetic modifications.

Epigenetic modifications are superimposed on the base sequence of DNA in multiple layers, which differ according to cell and tissue type.

These include:

- Modification of nucleosides in DNA by methylation and hydroxymethylation, usually at cytosine adjacent to guanine bases (CpG sites).
- Post-translational modification of histone proteins, such as by methylation and acetylation can contribute information to chromatin remodelling machinery. This determines how the chromatin is packaged, leading to raveling and unravelling of DNA, therefore genes and loci encoding non-coding RNAs become susceptible to transcription.
- Small non coding RNAs (such as micro RNAs) regulate gene expression by prompting mRNA degradation or modulating protein translation.

Figure reproduced with permission from Jones et al, Nature 2008 (Reid et al., 2008)

DNA methylation

This review will focus on DNA methylation as it is the most widely studied of epigenetic modifications and is the target of investigation in the ALPHABET project, though of course, the epigenetic processes of histone modifications and micro RNAs work in concert with DNA methylation to control gene expression (Curtis et al., 2018b). DNA methylation involves the transfer of a methyl group to the 5' carbon position of cytosine, creating 5-methylcytosine (5-mC) (Kumar et al., 1994). Though methyl marks can be added and removed throughout the lifecourse, it is a relatively stable epigenetic mark that can be transmitted through DNA replication during mitosis (Bird, 2002). Methylation of the cytosine base usually occurs within the dinucleotide sequence CpG, where a cytosine is immediately 5' to a guanine, with a phosphate group between them denoted by "p", although non-CpG methylation

is also prevalent in embryonic stem cells (Ramsahoye et al., 2000). A CpG site can either be methylated or unmethylated in an individual cell; however methylation is not completely uniform, even within a tissue or cell type. According to the level of methylation across a whole tissue where a particular site may be methylated or unmethylated in a large number of cells, a range of graded gene expression from 0% to 100% is possible (Gluckman et al., 2008).

Given their importance in transcriptional regulation, as would be expected, CpG dinucleotides are not distributed at random throughout the genome. They are clustered at the 5' end of genes in regions known as CpG islands, with hypomethylation generally associated with gene activation and hypermethylation with gene silencing (Song et al., 2005). Transcriptional repression in hypermethylated regions occurs through blocking of transcription factors binding to the DNA, or through recruitment of a myriad of other repressive factors, such as methyl CpG binding protein 2 (MeCP2), which in turn mediate local chromatin changes to impair transcription factor binding (Fuks et al., 2003).

CpG methylation patterns are largely established during embryogenesis, fetal and perinatal life. DNA methylation marks on the maternal and paternal genomes are largely erased on fertilisation (with the exception of the imprinted genes and other specific genomic regions), followed by a wave of *de novo* methylation within the inner cell mass just prior to blastocyst implantation (Okano et al., 1999, Santos et al., 2002). DNA methyltransferases (DNMT) 3a and 3b (Santos et al., 2002) catalyse *de novo* DNA methylation, and methylation patterns are maintained through mitosis in differentiated tissues by methylation of hemi-methylated DNA by DNA methyltransferase 1 (DNMT1) (Bacolla et al., 1999). However, DNA methylation patterns are not necessarily maintained throughout life as was initially thought: in 2009 the existence of another epigenetic modification, 5-hydroxymethylcytosine (5hmC), was described as present in high levels in neurons and embryonic stem (ES) cells (Tahiliani et al., 2009). 5-mC may be oxidised to 5hmC by the enzymes of the TET (Ten-Eleven-Translocation) family (Ito et al., 2011) and has been proposed to act as a specific epigenetic mark opposing DNA methylation, as well as a passive intermediate in the demethylation pathway (Guibert and Weber, 2013) (Wen and Tang, 2014).

Modifications to the epigenome through early life nutrition

Animal studies

Evidence is accruing that DNA methylation is modifiable, and a number of environmental factors such as nutrition, stress, placental insufficiency, endocrine disruptors and pollution, especially in early life, can alter the epigenome leading to long term phenotypic changes in the offspring (Feil and Fraga, 2011). A classic example of nutrition altering phenotype from the animal kingdom is that of the

honeybee. Though genetically identical, female honeybee larvae incubated in the presence of royal jelly predominantly develop into queen bees, while those incubated in the absence of royal jelly develop into sterile worker bees (Maleszka, 2008, Kucharski et al., 2008). Knockdown of DNA methyltransferase 3 (DNMT3), the major DNA methyl transferase in bees, was shown to increase the proportion of larvae developing into queen bees in comparison with sterile workers, indicating the role of DNA methylation in this process (Kucharski et al., 2008).

In mammals, a variety of studies have been carried out demonstrating the role of diet on phenotype, such as that of the agouti (A^y) mouse in which the coat colour is determined by the methylation status of an intracisternal-A particle (IAP) in the 5' upstream region of the agouti gene. When the pregnant female mice were fed a diet supplemented with folic acid, cobalamin, choline and betaine, a graded shift in coat colour in the offspring occurred from mainly yellow (agouti) to brown (pseudo-agouti) (Waterland and Jirtle, 2003). A change in methylation was detected as a result of this change in diet, with hypermethylation of seven CpG dinucleotides 600 bp downstream of the A^y IAP insertion site. Diet-induced changes in the offspring epigenome and metabolic health have been demonstrated in rats; feeding pregnant rats a protein rich (PR) diet was shown to induce hypomethylation of the glucocorticoid receptor (GR) and peroxisome proliferator activated receptor (PPAR) α promoters in the livers of juvenile and adult offspring; this was accompanied by an increase in GR and PPAR α expression and in the metabolic processes that they control (Lillycrop et al., 2005a, Lillycrop et al., 2007, Burdge et al., 2004). In comparison, the offspring of nutritionally restricted pregnant rats were shown to have increased levels of DNA methylation of PPAR α and the GR in the liver (Gluckman et al., 2007), suggesting that the effects of maternal nutrition on the epigenome of the offspring depend upon the nature of the maternal nutrient challenge, and that they may provide a means of adapting to an adverse environment (Gluckman et al., 2005). High fat (HF) diets versus low fat diets in mice have similarly been shown to induce different methylation patterns – for example hypomethylation of the μ -opioid receptor (MOR) and preproenkephalin (PENK) in the nucleus accumbens, prefrontal cortex, and hypothalamus of offspring mice from dams that consumed a HF diet during pregnancy (Vucetic et al., 2010). Various other examples can be found in mice, with sex-specific differences in terms of both exposure on the male or female parent and effects on the offspring; for example a paternal HF diet impacted glucose tolerance, pancreatic islet gene expression and hypomethylation of the anti inflammatory gene IL-13 receptor subunit alpha-2 (*Il13ra2*) in female offspring (Ng et al., 2010) (Barres and Zierath, 2016).

Human studies

Evidently, studies in animal models, where genetic variation in the subjects and diet pre- and post-pregnancy can be tightly controlled, have provided evidence of long term effects of maternal nutrition

on the offspring epigenome. Work in humans is more limited, though epidemiological studies on populations following periods of famine have provided important data. The study of the Dutch famine, or “Hunger Winter”, 1944, demonstrated alterations in the methylation of a number of genes in DNA isolated in whole blood from individuals whose mothers were exposed to famine. The timing of the nutritional constraint appeared to be important, as exposure to famine around the time of conception was associated with a small decrease in offspring CpG methylation of the imprinted *IGF2* gene and an increase in methylation of leptin, *IL-10*, *MEG3* and *ABCA4* (Tobi et al., 2009), while late gestation famine exposure had no effect on methylation. *In utero* experience of famine at any period in gestation led to an elevation in glucose levels and impaired glucose tolerance in adulthood, measured some 60 years after the famine exposure (Ravelli et al., 1998). Further links between type-2 diabetes risk and food restriction have been demonstrated in people with *in utero* famine exposure during the Chinese famine (1959-1961) (Li et al., 2017), the Ukrainian famine (1932-1933) (Lumey et al., 2015) and Austrian famines (1918-1919, 1938 and 1946-1947) (Turner et al., 2013).

In healthy human populations, studies of dietary supplementation have been shown to have long lasting effects, for example supplementation with folic acid 400 µg per day around the time of conception have also shown altered methylation of specific CpG sites in the *IGF2* gene in the peripheral blood cells of children (Steegers-Theunissen et al., 2009). Evidence also points towards plasticity in the human epigenome persisting into adulthood, as, for example, short-term high fat overfeeding in healthy young men was shown to induce methylation changes in over 6,000 skeletal muscle genes, with only partial reversal after 6-8 weeks of a normocaloric diet (Jacobsen et al., 2012).

DNA METHYLATION AND BONE DEVELOPMENT: POTENTIAL ROLES IN LATER LIFE OSTEOPOROSIS

Early growth and bone development

Osteoporosis is a common skeletal disorder characterised by low bone mass and loss of the normal bone microarchitecture, leading to increased bone fragility and therefore susceptibility to fracture (1993). With the globally ageing population, the burden of osteoporosis is increasing, with a consequent increase in fragility fractures worldwide (Oden et al., 2015). Such fractures typically occur at the hip, spine, wrist, humerus, pelvis, scapula and ribs. Most major osteoporotic fractures are associated with substantial morbidity and mortality – particularly for hip fractures, with an excess mortality of 10-20% in the first year after fracture, and a similar proportion requiring institutional care in the same period (Harvey et al., 2010a). Such fractures are very common - currently, the remaining lifetime risk of a fragility fracture in a UK woman aged 50 years is estimated at around 50%, and around 20% for a UK man (Harvey et al., 2010a, Curtis et al., 2016).

A strong predictor of later risk of osteoporotic fracture is an individual's peak bone mass, the maximum total skeletal mass accrued at the completion of skeletal development (Harvey et al., 2014a). Bone mass increases throughout fetal, infant, childhood and early adult life reaching a peak in the third to fourth decade, and this peak bone mass has been shown in mathematical modelling studies to be a more powerful predictor of the age of osteoporosis development than age at menopause or rate of subsequent age-related bone loss (Hernandez et al., 2003). Peak bone mass is of course partly explained by genetic factors as demonstrated by various genome wide association studies (Duncan et al., 2011, Mullin et al., 2016). Known genetic loci account for a small percentage of the overall variance, but next generation sequencing approaches may permit further characterisation of this 'missing heritability' such as the identification of rare genetic variants of stronger effect. Other factors that may contribute to links between early development and later health and disease include epigenetic effects and environmental influences.

Seminal work by David Barker and Clive Osmond in the 1980s, demonstrating that low birthweight is associated with increased risk of cardiovascular disease later in life (Barker and Osmond, 1986), was later applied to the field of osteoporosis by Cyrus Cooper. Weight in infancy was shown to correlate with adult bone mineral content in a variety of UK studies based in Bath, Sheffield and Hertfordshire (Cooper et al., 1995, Cooper et al., 1997, Gale et al., 2001). These findings, and those of several subsequent studies were confirmed in a meta-analysis showing that overall, each 1 kg increase in birthweight is associated with a 1.49g increase in bone mineral content (BMC) at the lumbar spine and 1.41g at the hip in adulthood. This effect was shown to be independent of adult weight and BMI (Baird et al., 2010). Furthermore, poor childhood growth and weight gain was associated with a greater risk of hip fracture in adulthood, further providing evidence that early nutrition is important for future skeletal development (Cooper et al., 2001, Javaid et al., 2011).

Mother-offspring cohorts have provided an opportunity for more detailed investigation into patterns of early growth (Harvey et al., 2014a, Harvey et al., 2012c, Harvey et al., 2010c) and specific maternal factors which might influence offspring development. The Southampton Women's Survey (SWS) (Inskip et al., 2005), a British prospective cohort of 12,583 initially non-pregnant women aged 20-34 years and their subsequent offspring (n=3156), is an example of a cohort which has provided important insights into the relationships between maternal factors and offspring bone mass. Low maternal fat stores, first pregnancy, smoking and high levels of physical activity during late pregnancy were all associated with reduced whole body BMC at birth (Harvey et al., 2010b), confirming findings from an earlier smaller study (Godfrey et al., 2001).

Epigenetic predictors of childhood bone health

Using an established discovery pathway, from array to candidate, epigenetic predictors of bone development have been discovered (Curtis et al., 2018a): analyses of DNA methylation in umbilical cord samples from the Princess Anne Hospital Cohort and the Southampton Women's Survey (Godfrey et al., 2011) have identified two key loci linked to bone outcomes: *CDKN2A* (Godfrey et al., 2011, Lillycrop KA, 2013, Murray et al., 2016, Curtis et al., 2017) and *RXRA* (Harvey et al., 2014d).

The *CDKN2A* locus encodes two cell cycle inhibitors: p14^{ARF} and P16^{INK4a}, which play roles in cellular senescence and ageing. The *CDKN2A* locus also encodes the long non-coding RNA ANRIL (antisense non-coding RNA in the *INK4* locus), a 3,834bp transcript which can negatively regulate *p16INK4a*. SNPs within the *CDKN2A* locus, particularly those located within *ANRIL* have been associated with cardiovascular disease, diabetes and frailty (Congrains et al., 2012), and DNA methylation at this locus has recently been demonstrated to vary with age (Bell et al., 2016). Previous studies have demonstrated links between perinatal *CDKN2A* methylation and later adiposity (Lillycrop et al., 2017); the functional relationships between fat and bone are well characterised, and mediated via both mechanical and endocrine pathways (Johansson et al., 2014). More recently, supported by the BBSRC project, we have shown that DNA methylation at CpG sites within the *CDKN2A* gene was associated with offspring bone mass at age 4 and 6 (Curtis et al., 2017) (Figure 2), with greater levels of methylation at the *CDKN2A* locus negatively associated with whole body minus head bone area (BA), bone mineral content (BMC) and areal bone-mineral density (BMD). This was confirmed in replication and combined data sets (all $p < 0.01$), with each 10% increase in methylation being associated with a decrease in BMC of 4-9 g at age 4 years ($p \leq 0.001$). Relationships were similar with 6 year bone mass, and functional investigations in a cell line demonstrated that methylation in this region could be important for transcription factor binding.

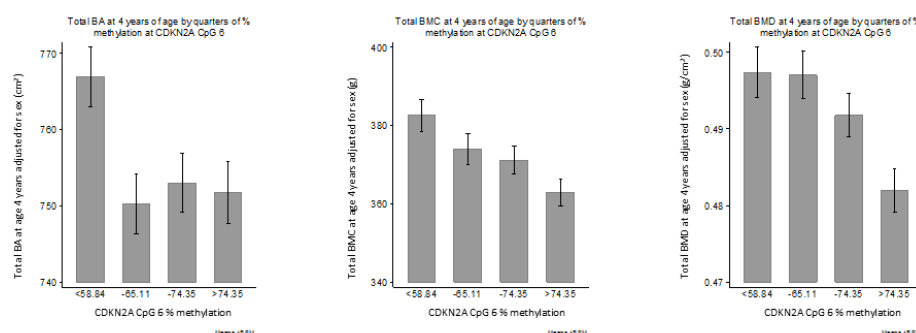


Figure 2

Perinatal methylation at CpG sites within the *CDKN2A* locus is associated with offspring bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) at age 4 years in the Southampton Women's Survey, $n = 555$. **Reproduced with permission** (Curtis et al., 2017)

Secondly, in the Southampton Women's Survey, methylation at several CpG sites around 2kb upstream from the promoter region of the retinoid X receptor-alpha (*RXRA*) gene in umbilical cord was correlated with lower offspring bone mineral content corrected for body size at four years old ($\beta = -2.1$ to -3.4g/SD , $p=0.002$ to 0.047), with the results supported by findings from a second independent cohort, the Princess Anne Hospital Study (Harvey et al., 2014d). Intriguingly, methylation at one CpG site was related to an estimate of free 25(OH)-vitamin D [25(OH)D] (Figure 3). *RXRA* is of particular interest in the context of bone health as it is an essential part of vitamin D signalling, forming a heterodimer with the vitamin D receptor (VDR) in the nuclear action of 1,25(OH)₂-vitamin D, as well as with other bone-active nuclear hormones. Vitamin D plays a central role in calcium and phosphate homeostasis, and severe vitamin D deficiency (VDD) can result in rickets, osteomalacia and neonatal hypocalcaemia. There is increasing evidence of a link between maternal gestational 25(OH)D status and offspring bone mass (Harvey et al., 2014b, Harvey et al., 2013, Harvey et al., 2006, Sayers and Tobias, 2009, Viljakainen et al., 2010, Zhu et al., 2014, Javaid et al., 2006), although not in all studies (Lawlor et al., 2013, Garcia et al., 2017), and that this association may be mediated partly through umbilical cord calcium concentrations (Javaid et al., 2006). Expression of a particular active placental calcium transporter was positively associated with neonatal bone mass (Martin et al., 2007), and with regulation by 1,25(OH)₂-vitamin D suggested in experimental studies (Kip and Strehler, 2004). More recently, the hypothesis that maternal gestational supplementation with vitamin D would lead to increased offspring bone mass was tested in the MAVIDOS Maternal Vitamin D Osteoporosis Study, a randomised, placebo-controlled, double-blind trial of 1000 IU vitamin D versus placebo daily from 14 weeks gestation until delivery of the infant (Cooper et al., 2016, Harvey et al., 2012b). The results of this trial have suggested that such a supplementary approach leads to improved offspring bone mineral content (of around 0.5 SD) compared with placebo, for neonates born in the winter months, when background 25(OH)D concentrations are lowest (although not in the population overall) (Cooper et al., 2016). Subsequent work has identified interactions with both environmental and genetic factors in the achieved 25(OH)D in response to treatment (Moon et al., 2016, Moon et al., 2017); mechanistic investigations using placental samples have identified novel relationships between vitamin D and placental nutrient transport (Cleal et al., 2015).

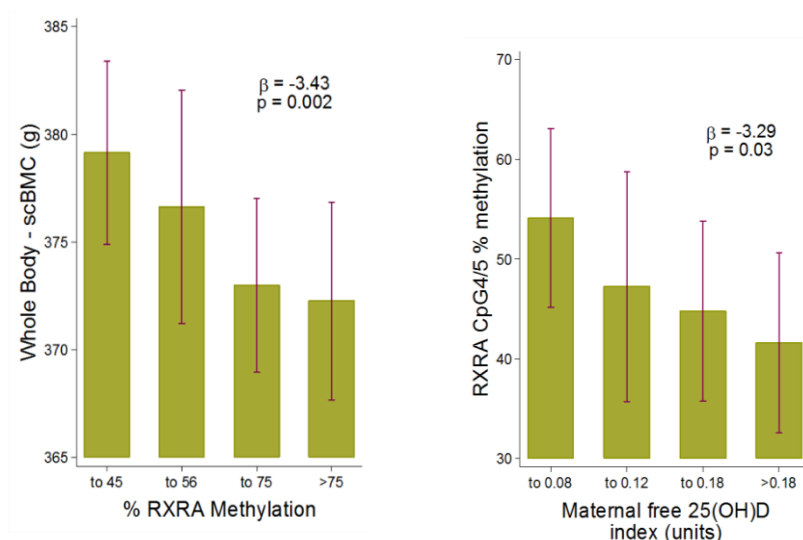


Figure 3

Percent DNA methylation at *RXRA* & offspring size corrected bone mineral content (a); maternal 25(OH)D & *RXRA* DNA methylation, $n = 230$ (b). scBMC, size-corrected Bone Mineral Content. Adapted with permission from Harvey et al, 2014 (Harvey et al., 2014d)

These candidate-focused studies contrast with findings from a recent investigation using the Illumina HumanMethylation450 BeadChip, an array covering methylation marks at over 450,000 CpG sites across the genome. Amongst 819 mother-offspring pairs in the Norwegian Mother and Child Cohort and 597 mother-offspring pairs in the Avon Longitudinal Study of Parents and Children (ALSPAC), there were no convincing associations between maternal mid-pregnancy 25(OH)D status and methylation profile in cord blood DNA. Additionally, use of the same array technology in blood DNA from 5515 adults of European descent identified only one CpG site associated with femoral neck bone mineral density. However these studies do not specifically address links between perinatal epigenetic marks and offspring bone development and there are a number of important considerations here including (1) the tissue specificity of epigenetic marks and the use of blood DNA compared with perinatal tissues such as umbilical cord which contain mesenchymal stem cells that have potential to develop into target tissues such as bone, muscle and fat; (2) despite coverage of >450,000 CpG sites, the array targets a tiny percentage of the number of potentially methylated CpG sites across the genome; (3) although the variance explained by molecular phenotype is generally greater than that explained by fixed genetic variation, the sample sizes of these consortium-based studies have been small compared with what has recently been possible with genetic analysis. For example, a genome-wide association study for heel bone mineral density in the UK Biobank cohort using the then available subset of 142,487 individuals yielded 307 loci, including 153 previously unreported sites (Kemp et al., 2017), representing a step change in the number of sites identified compared with previous studies, for example comprising combined cohorts of around 50,000 individuals, identifying 56 loci (Estrada et al., 2012).

PLANNED WORK

Through the BBSRC funded component of the ALPHABET project, the aim is to investigate dietary and epigenetic predictors of later bone health, and to thus address questions raised by the evidence base to date. The BBSRC funded component comprises two parts: firstly the generation of the epigenetic data, led by Professor Caroline Relton at the MRC Integrative Epidemiology Unit, University of Bristol; secondly the analysis of links between early diet, perinatal epigenetic marking and offspring bone health, led by Professor Nicholas Harvey at the MRC Lifecourse Epidemiology Unit, University of Southampton; thirdly the application of causal analysis methods to improve the evidence base for causal pathways rather than merely observed associations. The following briefly summarises the planned work:

Generation and collation of methylation data

DNA methylation profiles generated by the Illumina Infinium HumanMethylation450 BeadChip are available for ALSPAC (1000 mother-child pairs, for mothers during pregnancy and 8 years after delivery, and for children at birth and at 7 years and 15-17 years) (Relton et al., 2015), Repro_PL (150 mother-child pairs, for mothers during pregnancy and birth) and Generation R (1500 birth, 500 each at 6 years and 9 years). Offspring DNA from birth are from cord blood; follow-up and maternal DNA are from peripheral blood. Led by the University of Bristol, framework and scripts are being designed and distributed for analysing DNA methylation profiles from individual datasets to allow meta-analyses of summary statistics from each “discovery” dataset. A series of diet-methylation and methylation-phenotype analyses are being performed. Mediation analysis, and 2-sample Mendelian randomization where possible, of identified “hits” will test whether methylation may be on the causal pathway. Currently unprofiled cohorts for which DNA is available may be used for replication of specific CpGs identified in the discovery set, and in further analyses epigenetic age of mothers and children will be investigated.

Early diet, epigenetic marks and offspring bone health

Using the ALSPAC, SWS and ROLO cohorts, and led from the University of Southampton, this work package explores 1) relationships between maternal and childhood dietary indices [Dietary Approaches to Stop Hypertension (DASH) and Dietary Inflammation Index (DII) (Fung et al., 2008, Shivappa et al., 2014)] and child bone indices by DXA; and 2) perinatal epigenetic marks from Illumina 450K/850K array analysis (maternal blood and cord blood) and DXA bone outcomes; controlling for potential covariates. Where offspring biological samples are available later in childhood, differences in epigenetic profile will be related to maternal and perinatal profiles, and whether they relate to current bone mass or can be explained by offspring body composition, physical activity and diet.

Adjunctive data may be obtained from the MAVIDOS trial, in which Illumina 850K analysis has been undertaken on umbilical cord tissue, together with specific validation of candidates using techniques such as Pyrosequencing on SWS and MAVIDOS cohorts.

CONCLUSION

The BBRSC-funded musculoskeletal work forms just a part of the multinational collaborative ERA-HDHL Biomarkers ALPHABET project on maternal nutrition, child health and epigenetics, and which provides a unique opportunity to improve maternal and offspring health for the future through state-of-the-art science. With the prevalence of many chronic non-communicable diseases increasing worldwide, population level strategies are needed to reduce the future burden of these conditions. In ALPHABET, the developmental origins of disease paradigm is being investigated through the study of DNA methylation, to understand interactions between maternal nutrition, offspring phenotype and future health, across thousands of mother-child pairs throughout Europe. In addition to informing mechanism understanding, the findings from the ALPHABET study should inform potential novel dietary interventions, identify epigenetic signals that may constitute biomarkers of future disease risk, and thus targeted and population-wide strategies to optimise periconception and early postnatal environment and consequently reduce the burden of chronic non-communicable conditions such as osteoporosis and cardiometabolic disease in future generations.

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Conflicts of Interest

No conflicts of interest have been declared.

APPENDIX: Details of the cohorts contributing data to the ERA-HDHL ALPHABET project

A number of European longitudinal birth cohorts currently collaborate as part of the ALPHABET project, providing important insights into the influence of maternal exposures on offspring health. Here we summarise the cohorts and a selection of relevant findings leading up to the ALPHABET project.

Avon Longitudinal Study of Parents and Children (ALSPAC)

ALSPAC (Boyd et al., 2013) (Fraser et al., 2013) is a prospective pregnancy cohort, recruiting 13,761 women resident in England during 1991-92. It has tested a large number of associations between early life dietary exposures and later health outcomes (Emmett and Jones, 2015, Emmett et al., 2015). DNA methylation profiles are available for 1000 mother-children pairs during pregnancy, at birth and up to 18 years follow-up. Relevant investigations include those of associations of DNA methylation with birthweight and gestational age (Simpkin et al., 2016, Simpkin et al., 2015), maternal pre-pregnancy BMI, gestational weight gain (Sharp et al., 2015), serum vitamin D (Suderman et al., 2016) and prenatal smoking (Richmond et al., 2015) and investigation of DNA methylation as a causal mechanism for these associations (Richmond et al., 2016) (Kupers et al., 2015).

The Southampton Women's Survey (SWS)

The Southampton Women's Survey (SWS) (Inskip, 1999), recruited 12,583 women aged 20 to 34 years between 1998-2002 in Southampton, UK; of these 3,158 children were born between 1998-2007 and followed-up to age 10-13. Importantly, maternal data are available pre-conception. The main aims of the study are to characterise maternal diet, lifestyle and intrauterine effects on offspring fetal growth and pathways leading to poor health outcomes. Dietary factors during pregnancy have been associated with childhood adiposity, bone, muscle and cognitive development (Okubo et al., 2014, Gale et al., 2009, Harvey et al., 2012a, Harvey et al., 2014c), and between maternal adiposity (Pike et al., 2013) or vitamin D status (de Jongh et al., 2014) and offspring respiratory health. DNA methylation analysis demonstrated associations with later bone mineral content (Harvey et al., 2014d, Curtis et al., 2017), adiposity (Lillycrop et al., 2017) and neurocognitive function and behaviour (Lillycrop et al., 2015), providing support for a role of epigenetic processes in mediating the long-term consequences of early life environment on health.

The Generation R Study

The Generation R Study (Jaddoe et al., 2006, Kooijman et al., 2016) is a population-based prospective cohort study of ~10,000 pregnant women and their children in the Netherlands. Its aims are to identify both genetic and early environmental causal pathways relating to growth, development and health from fetal life to young adulthood. Relevant findings include associations between maternal diet during early pregnancy and childhood body composition, bone mass, and risk of wheezing and eczema at 4-6 years (van der Valk et al., 2013) (Braun et al., 2015). Higher plasma folate and vitamin B12 concentrations, or high folic acid intake of mother during pregnancy was associated with lower offspring body weight and BMI and increased risk of eczema at 4-6 years (van der Valk et al., 2013) (Braun et al., 2015) (Kiefte-de Jong et al., 2012). DNA methylation studies (subgroup n=1,232), through either candidate gene or epigenome-wide association studies, showed that maternal plasma folate concentrations and smoking during pregnancy impacts DNA methylation in newborns (Bouwland-Both et al., 2015) (Joubert et al., 2016a) (Joubert et al., 2016b).

The EDEN mother-child cohort study

Based in France, the EDEN mother-child cohort study of 2002 pregnant women investigates prenatal and early postnatal determinants of fetal and postnatal growth, adiposity development, respiratory and bone health and neurodevelopment (Heude et al., 2016). Lines of investigation include diet, environmental pollutants, socioeconomic and psycho-emotional factors. In the study, detailed questionnaire and clinical data on phenotypes and exposures and biological samples were collected from pregnancy until the child was 8 years old. Relevant findings from the ALPHABET project include tracking of dietary patterns from infancy to pre-school age (Lioret et al., 2015), associations between maternal dietary factors (caffeine and fatty acids) with offspring IQ (Galera et al., 2016), neurodevelopment (Bernard et al., 2013) and DNA methylation (Azzi et al., 2014), and between gestational weight change and offspring adiposity (Diouf et al., 2014) (Jacota et al., 2017).

The Polish Mother and Child Cohort (Repro_PL)

The Polish Mother and Child Cohort (Repro_PL), is a multicentre prospective cohort study of 1800 mother-child pairs, in which the children have been followed up to age 7. It was established in 2007 with the aim of evaluating the contribution of environmental factors to pregnancy outcomes, children's health and neurodevelopment (Polanska et al., 2011). Findings to date suggest that maternal lifestyle, micronutrient and vitamin D status during pregnancy and child environment after birth have significant impacts on child health and psychomotor development (Polanska et al., 2016) (Stelmach et al., 2014, Polanska et al., 2015) (Stelmach et al., 2015).

The Lifeways Cross-Generation Cohort Study

Based in the Republic of Ireland, the Lifeways Cross-Generation Cohort Study recruited 1100 pregnant women in 2001 and is one of very few worldwide containing data on grandparents of both lineages (Kelleher et al., 2014). The children have been followed up at age 5 and 9 years, with physical examinations and linkage to hospital data and general practice records. Consistent familial and cross-generational associations, particularly along the maternal line, have been reported between parental and grandparental health status and child outcomes, including BMI and asthma (Kelly et al., 2014) (Murrin et al., 2012).

The ROLO Study

The ROLO study (Randomised cOntrol trial of LOw glycaemic index diet *versus* no dietary intervention to prevent recurrence of fetal macrosomia), also based in the Republic of Ireland, recruited 800 women. In the low glycaemic index diet intervention group, reduced gestational weight gain and improved maternal glucose intolerance was seen. However, no difference in offspring weight or BMI at birth or at 6 month follow-up was found between groups (Horan et al., 2016) (Walsh et al., 2012). Five year follow up of the mothers and children is currently underway.

Secondary analysis of the ROLO cohort examined the effect of dietary calcium, dietary vitamin D and seasonal variation in serum 25(OH)D on a marker of bone resorption (urine cross-linked N-telopeptides of type I collagen (uNTX) during pregnancy (O'Brien et al., 2017). In late pregnancy, during winter months when 25(OH)D is inadequate, intakes of dietary calcium <1000 mg/day were associated with significantly increased bone resorption. Additional dietary calcium is associated with reduced bone resorption in late pregnancy, with greater effect observed in winter pregnancies.

The PEARS study

Building on findings from the ROLO study, the PEARS study (Pregnancy, Exercise and Nutrition Research Study with smart phone app support) of 500 women in the Republic of Ireland will assess the impact of a 'healthy lifestyle package' involving targeted, low GI nutritional advice plus daily physical activity delivered before 18 week's gestation, together with a smart phone app to provide ongoing healthy lifestyle advice and support throughout pregnancy. Biological samples from early and late pregnancy as well as cord blood will be collected and a wide range of maternal and fetal health outcomes measured (Kennelly et al., 2016). The study aims to evaluate the effectiveness of a smart

phone App intervention, grounded in behaviour change theories and techniques, with the aim of preventing gestational diabetes mellitus in an overweight or obese pregnant population.

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